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**(54) [Title of the Invention]** New Use

(57) [Abstract]

[Structure] A nitric oxide production inhibitor characterized by the use of 2-iminopiperidine or one of its pharmacologically acceptable salts as the active ingredient.

[Effect] 2-iminopiperidine and its pharmacologically acceptable salts have utility because of their strong inhibition of nitric oxide production and their low toxicity.

[Scope of the Invention]

[Claim 1] A nitric oxide production inhibitor characterized by the use of 2-iminopiperidine or one of its pharmacologically acceptable salts as the active ingredient.

[Detailed Explanation of the Invention]

[0001]

[Field of Industrial Use] This invention relates to methods for the manufacture of 2-iminopiperidine and its pharmacologically acceptable salts or nitric oxide (NO) production inhibitors containing them as their active ingredients, and can be used in the field of medicine.

[0002]

[Prior Art] 2-iminopiperidine hydrochloride is a widely-known substance which is marketed as a synthetic reagent , but methods of manufacture of 2-iminopiperidine or its pharmacologically acceptable salts by means of fermentation by microorganisms as well their ability to inhibit the production of nitric oxide (NO) are unknown.

[0003]

[Structure of the Invention] This invention relates to methods for the manufacture of 2-iminopiperidine and its pharmacologically acceptable salts or nitric oxide (NO) production inhibitors containing them as their active ingredients. 2-iminopiperidine can be manufactured not only through chemical synthesis, but, for example, using streptomyces atroolivaceus No. 70757, a microorganism newly isolated as described below by the inventors from a soil sample obtained in New Zealand. The microbiological properties of streptomyces atroolivaceus No. 70757 are given below.

[0004] The Microbiological Properties of No. 70757

No. 70757 was isolated from a soil sample obtained in New Zealand. The culture medium and the method used to investigate the morphology, culture characteristics and physiological characteristics of this strain were mainly based on Shirling and Gottleib [(1)] and Waksman.[(2)] The various cultures were observed after 14 days at 30 degrees Celsius. Observations of morphology were carried out using optical and scanning electron microscopes after cultivation using yeast extract/malt extract agar, oatmeal agar, and non-organochlorine agar. Carbon source utilization made use of the culture medium of Pridoham and Gottlieb [(3)]. Color names were taken from the Methuen Handbook of Colour.[(4)] The amino acids of cell walls were according to Becker et al.[(5)] and Yamaguchi.[(6)] Freeze-dried samples of this strain have been deposited in the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology (1-1-3 Higashi, Tsukuba-shi, Ibaraki-ken). (Deposit Number: FERMBP-4972; Date of Deposit: January 18, 1995)

[0005] (1) Morphological Characteristics

The vegetative mycelium develops well, and branches irregularly but does not separate. The aerial mycelia that extend from the vegetative mycelium exhibit simple branching and form long spore chains. The shape of the aerial mycelia was classified as RF according to the Pridham et al.[(7)] classification of straight, bent and ridged. Single spore chains consisted of 20 or more spores. The spore surfaces were smooth, and they were cylindrical in shape, with dimensions of 0.5-0.7 x 0.5-0.7 $\mu$m. Sclerota, sporangia and zoospores were not observed.

(2) Culture Characteristics

Results are shown in Figure 1. The aerial mycelia ranged from light gray to gray in color, and the vegetative mycelia displayed dark gray, olive and olive gray colors. Pigments inside of mycelia were not pH-sensitive. Soluble pigments and melanoid pigments were not observed.

(3) Cell Wall Type

The results of the analysis of the entire fungus body were able to confirm the presence of LL-diaminopimelic acid. Thus, the cell walls of this strain can be considered to be Type I.

(4) Physiological Characteristics

As seen in Figure 2, the strain was capable of utilizing many carbon sources, and hydrolysis of starch was positive.

(5) Identification

Strain No. 70757 can be judged to belong to the genus streptomyces from the results of morphological observations and chemical analysis.[(7)] Additionally, this strain was compared to the species of the genus streptomyces that are found in the literature.[(8-12)] We found that the characteristics given for Streptomyces atroolivaceus were almost identical to those of this strain. Thus, this strain was named Streptomyces atroolivaceus No. 70757.

[0006]

[Figure 1]

Figure 1: Culture Characteristics of No. 70757

| Culture | Culture Properties |
|---|---|
| Yeast Extract/Malt Extract Agar (ISP-2) | G: Normal<br>A: Poor, light gray (1D1)<br>R: Dark gray (1F1)<br>S: None |
| Oatmeal Agar (ISP-3) | G: Normal<br>A: Poor, gray (1E1)<br>R: Olive (2F3)<br>S: None |
| Non-Organochlorine/Starch Agar (ISP-4) | G: Normal<br>A: Good, gray (1E1)<br>R: Olive gray (2F4)<br>S: None |
| Glycerol/Asparagine Agar (ISP-5) | G: Good<br>A: Abundant, gray (1E1)<br>R: Olive (3F5)<br>S: None |
| Peptone/Yeast/Fe Agar (ISP-6) | G: Poor<br>A: None<br>R: Colorless<br>S: None |
| Tyrosine Agar (ISP-7) | G: Good<br>A: Normal, gray (1E1)<br>R: Olive brown (4F3)<br>S: None |

G: Growth; A: Aerial Mycelia; R: Color of Bottom of Growth; S: Soluble Pigment
The code following the color of the bottom of the growth is taken from (4) in the works cited

[0007]
[Figure 2]

Figure 2: Carbon Source Utilization of No. 70757

| Carbon Source | Growth |
|---|---|
| D-Glucose | + |
| Sucrose | + |
| D-Xylose | + |
| D-Fructose | ± |
| L-Rhamnose | + |
| Raffinose | + |
| L-Arabinose | + |
| Inositol | – |
| D-Mannitol | ± |

+: Utilizes ±: Utilizes Slightly
-: Does not Utilize

[0008] Works Cited

(1) Shirling, E. B. and D. Gottlieb: Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol. 16, 313–340, 1966

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[0009] It should be understood that the manufacture of 2-iminopiperidine and its pharmacologically acceptable salts is not limited to the use of the specific microorganisms described in these specifications, which are named simply for the purposes of explanation. This invention also includes the use of all mutants, including artificial and natural mutants, capable of manufacturing 2-iminopiperidine or its pharmacologically acceptable salts that can be obtained through mutation of the microorganism described using X rays, ultraviolet rays, N-methyl-N'-nitro-N-nitrosoguanidine, 2-aminopurine, or other methods.

[0010] The manufacture of the 2-iminopiperidine and its pharmacologically acceptable salts related to this invention can be caused by injecting microbes capable of manufacturing said substance (for example, No. 70757) into a culture medium which contains carbon and nitrogen sources which they can make use of and culturing them under aerobic conditions (for example, in a shaken culture or an aerated spinner culture). The use of glucose, sucrose, xylose, rhamnose, arabinose, mannitol, cottonseed cake, starch, modified starch, fructose, glycerin or other carbohydrates as carbon sources is recommended. The use of oatmeal, yeast extract, peptone, gluten meal, cottonseed cake, cottonseed oil cake, soybean cake, corn steep liquor, dried yeast, wheat germ, peanut cake, or chicken bone/meat meal as nitrogen sources is recommended, but organic and inorganic nitrogen compounds such as ammonium salts (for example, ammonium nitrate, ammonium sulfate and ammonium phosphate), urea, and amino acids may also be used advantageously.

[0011] These carbon and nitrogen sources can be used advantageously in tandem, but it is not necessary to use pure products. Non-pure products may include ingredients such as growth factors and microelements, as they may be beneficial. If necessary, inorganic salts such as the following may be added to the culture: sodium carbonate, potassium carbonate, calcium carbonate, sodium phosphate, calcium phosphate, sodium chloride, calcium chloride, sodium iodide, calcium iodide, magnesium salts (such as magnesium sulfate), copper salts, cobalt salts. In particular, when the culture medium foams strongly, liquid paraffin, animal fat, vegetable oil, mineral oil, adecanol LG-109 (made by Asahi Denkakagyou K.K.), silicon, or other substances may be added.

[0012] In order to industrially manufacture the end product in large quantities, it is desirable to use an aerated spinner culture in the manner of other fermented products. A shaken culture equipped with a flask is suitable for the manufacture of small quantities. Additionally, in order to prevent delayed growth of the microorganism during the production of 2-iminopiperidine or its pharmacologically acceptable salts when the culture is being carried out in a large tank, it is advisable to first inject the producing microorganism into a comparatively small amount of the culture medium, cultivate it, and then move the culture to a large production tank for production culture. In this case, the culture media used for the preliminary culture and the production culture may be the same, but if necessary, they may be different as well.

[0013] It is advisable that cultivation be carried out under conditions of aerated agitation, using a suitable previously-known method such as agitation using a propeller or other mechanism, the rotation or shaking of the fermenter, pump processing, or the intake of air. The temperature of the culture can be changed freely within the range within which microorganisms that produce 2-iminopiperidine or its pharmacologically acceptable salts produce these substances, but cultivation is ordinarily carried out between 1 and 40 degrees Celsius, ideally between 14 and 36 degrees. The length of cultivation can differ with the conditions and quantity of the culture, but it is ordinarily between one day and one week. After the fermentation is completed, the 2-iminopiperidine or the pharmacologically acceptable salt thereof which is the product is extracted. For example, the fungus body is extracted with water and/or an organic solvent, or it is broken down mechanically and/or using ultrasonic waves or another previously known method and extracted with water and/or an organic solvent, and it is collected and refined according to the state of the art. When a liquid culture is used, it is collected and refined directly according to the state of the art.

[0014] The method of collection and refining can include solvent extraction using water, an organic solvent, or a mixture thereof, chromatography using an ion exchange resin or other method, or recrystallization from a single or mixed solvent, and suitable methods can used alone or in combination. The pharmacologically acceptable salts of 2-iminopiperidine include those salts which are ordinarily non-toxic, for example salts of organic acids (such as acetate, maleate, tartrate, methanesulfonate, benzene sulfonate, formate, toluenesulfonate or trifluoroacetate), salts of inorganic acids (such as acetate, hydrobromate, sulfate, or phosphate), and amino acids (such as aspartic acid or glutamic acid).

[0015] The 2-iminopiperidine and its pharmacologically acceptable salts in this invention have the effect of inhibiting the production of nitric oxide (NO), and are of use as the active ingredients in nitric oxide inhibitors. Nitric oxide inhibitors

using this invention are effective in the prevention and treatment of septic shock, type I diabetes, diabetic kidney disease, diabetic retinopathy, diabetic neuropathy, ulcerative colitis, chronic colitis, glomerulonephritis, cerebral infarction, chronic rheumatoid arthritis, osteoarthritis, gout, migraine, neuritis, osteoporosis, STE (systemic lupus erythematosus), organ transplants, asthma, ARDS (adult respiratory distress syndrome), postherpetic neuritis, myocarditis, heart failure, synovitis, metastasis of cancer, and Alzheimer's disease. Next, the outstanding ability to inhibit the production of nitric oxide (NO) displayed by the 2-iminopiperidine of this invention and its pharmacologically acceptable salts is described using Test Example 1.

[0016] Test Example 1

(1) Nitric Oxide (NO) Inhibition Activity Using J774.1 Cells

J774.1 cells (of mouse macrophage origin) suspended in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) were dispensed through a 96-hole plate (made by Corning), and cultured in a 37 degree Celsius 5% carbonic acid gas culture incubator until the number of cells per well reached 6-8x$10^4$. Afterwards, they were transferred to a 10% FBS:DMEM culture medium containing 1 g/ml lipopolysaccharide (LPS) and 4 U/ml Interferon-$\gamma$ (INF-$\gamma$), which also included the test compound and had been previously diluted to the target concentration, and were cultivated for an additional twenty hours. 90 $\mu$l of the cell conditioned medium obtained was added to 100 $\mu$l of Greiss reagent (1% sulphanilimide and 0.1% naphthylethylenediamine in a 2.5% aqueous solution of phosphoric acid), nitrite levels were measured as metabolites of NO through measurement of absorption at 550 nm, and the inhibition of nitric oxide production was calculated.

(2) Test compound: 2-iminopiperidine hydrochloride

(3) Results:

[Figure 3]

| Test compound ($\mu$g/ml) | inhibition activity (%) |
|---|---|
| 32 | 100 |

Additionally, the 2-iminopiperidine and its pharmacologically acceptable salts of this invention excel in safety, as is described using Test Example 2.

[0017] Test Example 2

Acute toxicity test

(1) Test compound: 2-iminopiperidine hydrochloride

(2) Testing methods and results: Five-week-old ICR mice were orally administered the test compound dissolved in physiological saline using oral probes and observed from the administration of the medication to the seventh day, and any cases of death were noted. As a result, absolutely no cases of death could be observed during one week of care and observation of mice which had been orally administered 320 mg/kg, 100 mg/kg, 32 mg/kg, 10 mg/kg and 3.2 mg/kg.

[0018] It is possible to administer the 2-iminopiperidine and its pharmaceutically acceptable salts of this invention, which are active ingredients in nitric oxide production inhibitor, by themselves, but they will ordinarily be administered as various compound preparations. Various examples of forms that preparations can take include oral preparations such as solutions, emulsions, suspensions, capsules, granules, powders, tablets and syrups, external and topical preparations such as ointments, eye drops and nose drops, inhalants, injectables and suppositories. These preparations can be made according to the state of the art using fillers such as sucrose, starch, mannitol, sorbitol, lactose, fructose, glucose, cellulose, talc, calcium phosphate and calcium carbonate, binders such as cellulose, methylcellulose, hydroxypropyl cellulose, polypropylpyrrolidone, gelatin, gum arabic, polyethylene glycol, sucrose and starch, disintegrators such as starch, carboxymethylcellulose, hydroxypropyl starch, sodium bicarbonate, calcium phosphate, carboxymethylcellulose calcium and calcium citrate, lubricants such as magnesium stearate, talc and sodium lauryl sulfate, flavoring agents such as citric acid, menthol, glycene, sorbitol and orange powder, preservatives such as sodium benzoate, sodium sulfite, methyl paraben and propyl paraben, stabilizers such as citric acid, sodium citrate, sodium edetate and acetic acid, suspending agents such as methylcellulose, polyvinylpyrrolidone and aluminum stearate, dispersants such as hydroxypropyl methylcellulose, solvents such as hydrochloric acid, emulsifiers such as sodium monostearate, flavors such as lemon essence premia, antiseptics such as benzalkonium chloride, and bases such as cocoa butter, polyethylene glycol, microcrystalline wax, bleached beeswax, liquid paraffin and white petroleum jelly. The amount of administration for preparations of this invention will differ according to the age, weight and condition of the patient and the method of administration, but the amount of the active ingredient 2-iminopiperidine or its pharmaceutically acceptable salts that is administered will ordinarily be in the range of 1 to 300 mg, preferably within the range of 1 to 100 mg, and can be administered orally or non-orally. The invention is explained below using working examples.

[0019] Working Example 1

The ingredients in the preparation below are mixed according to the state of the art to make an injectable preparation.

[Figure 4]

Contents of Injectable Preparation

| | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 100 mg |
| Solvent - Water for injection | As needed |
| Total volume | 10 ml |

[0020] Working Example 2

The ingredients in the preparation below are mixed according to the state of the art to make an inhalant. [Figure 5]

Contents of Inhalant Preparation

| | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 12 mg |
| Filler - Lactose | 88 mg |
| Total volume | 100 mg |

Working Example 3

The ingredients in the preparation below are mixed according to the state of the art to make an injectable. [Figure 6]

Contents of Injectable (Injectable Preparation for Drip Infusion) Preparation

| | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 5 mg |
| Solvent - Water for injection | As needed |
| Total volume | 5 ml |

[0021] Working Example 4

The ingredients in the preparation below are mixed and compressed according to the state of the art to make tablets. [Figure 7]

Contents of Tablet Preparation

| | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 50 mg |
| Disintegrator - Carboxymethylcellulose calcium | 3 mg |
| Bonding agent - Hydroxypropyl cellulose | 1 mg |
| Filler - Crystallized cellulose | As needed |
| Lubricant - Magnesium stearate | 2.5 mg |
| Total volume | 90 mg |

[0022] Working Example 5

The ingredients in the preparation below are mixed according to the state of the art to make ointment. [Figure 8]

Contents of Ointment Preparation

| | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 5 g |
| Base - Bleached beeswax | 5 g |
| Base - Liquid paraffin | 23.9 g |
| Base - White petroleum jelly | 40 g |
| Base - Microcrystalline wax | 2 g |
| Emulsifier - Glycerine monostearate | 2 g |
| Solvent - Purified water | As needed |
| Total volume | 100 g |

[0023] Working Example 6

The ingredients in the preparation below are mixed according to the state of the art to make syrup. [Figure 9]

| Contents of Syrup Preparation | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 500 mg |
| Sweetener - 70% sorbitol solution | 30 g |
| Preservative - Sodium benzoate | 0.1 g |
| Flavor - Lemon essence premia | 0.04 g |
| Solvent - Purified water | As needed |
| Total volume | 100 ml |

[0024] Working Example 7

The ingredients in the preparation below are mixed according to the state of the art to make nose drops. [Figure 10]

| Contents of Nose or Eye Drop Preparation | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 50 mg |
| Antiseptic - Benzalkonium Chloride | 0.01 mg |
| Stabilizer - Sodium edetate | 0.01 mg |
| Solvent - Water for injection | As needed |
| Total volume | 10 ml |

[0025] Working Example 8

The ingredients in the preparation below are mixed according to the state of the art to make suppositories [Figure 11]

| Contents of Suppository Preparation | |
|---|---|
| Main ingredient - 2-iminopiperidine hydrochloride | 20 mg |
| Base - Polyethyl glycol (PEG) | 980 mg |
| Total volume | 1000 mg |

[0026] Working Example 9

160 ml of a liquid culture medium composed of 1% cornstarch, 1% glucose, 0.5% peanut cake, 0.5% soybean cake 0.5% dried yeast and 2% CaC03 is added to each of four 500-ml Erlenmeyer flasks, and they are sterilized for 30 minutes at 121 degrees Celsius. Each of the culture media is inoculated once with a diagonal culture of streptomyces atroolivaceus No. 70757 using a platinum loop, and they are cultivated while shaking for three days at 30 degrees Celsius. Next, 20 l of a liquid culture medium composed of 2% sucrose, 1% modified starch, 0.5% corn steep liquor, 0.5% peptone 1% peanut cake, 0.05% $MgSO_4$-$7H_2O$, 0.02% $CaCO_3$ ($CaCO_3$ is added after the pH of the liquid culture medium is adjusted to 6.8 using 6-N-sodim hydroxide) and 0.05% antifoaming agent (adecanol LG-109, manufactured by Asahi Denkakagyou K.K.) is added to each of two 30-l jar fermenters, and after they are sterilized for 30 minutes at 121 degrees Celsius, each is inoculated with 2 of the above-mentioned cultures and cultivated under aerated spinning for three days at 30 degrees Celsius. After cultivation is complete, a filtrate adjuvant is added to the above-mentioned cultures and is filtered to obtain 35 l of filtrate. 4 l of this filtrate is passed through a negative ion exchange resin (Dowex 1 x 2; $OH^-$ type) column. This solution is adjusted to 6.5 pH using 6N-HCl, and after washing with 10 l of water adsorbed into a 2-l activated charcoal column, it is eluted with 0.01 N-HCl. The elution domain (22l) is adjusted to pH 6.0 with 6-N-NaOH and is adsorbed into a 11 CM-Sephadex C-25 positive ion exchange resin (manufactured by Pharmacia Japan) (equilibrated with 0.2 M-$NaH_2PO_4$) column. After this column is washed with 4 l of ion exchange water, the active substance is eluted with 0.1 M-$NaH_2PO_4$. After 3.3 l of this elution domain is adsorbed into a column of 1-l Sepabeads SP-207 (manufactured by Mitsubishi Kasei K.K.) adsorption resin, it is washed with 3 l of 0.1 M-$NaH_2PO_4$ and 4 l of ion exchange water, and the active substance is eluted with a 0.0025 N-HCl-50% methanol solvent. After the active fraction is concentrated in a vacuum and the methanol is removed, it is freeze dried. After 50 mL of methanol is added to the freeze-dried product to remove insoluble materials, the active fraction is concentrated, dried and hardened in a vacuum, yielding 450 mg of an oily substance. This oily substance is dissolved in acetonitrile (30 ml), the precipitate is removed, and a white powder (230 mg) is obtained through concentration in a vacuum. The physiochemical properties of the white powder obtained are as given below.

[0027] (1) Molecular Formula:

$C_5H_{10}N_2$-HCl

(2) FAB-MS:

FAB-MS (m/z): 99 (M+H free bodies)

(3) Elemental Analysis:

Calculated Values (%) ($C_5H_{10}N_2$-HCl)

C: 44.61; H: 8.24; N: 20.81; Cl: 26.34

Actual Values (%) C: 44.15; H: 8.64; N: 20.37; Cl: 24.92

(4) Ultraviolet Absorption Spectrum:

FT-IR (KBr): 3300-2700, 1680, 1530, 1420, 1360, 1330, 1170, 990 $cm^{-1}$

(5) $^1H$ Nuclear Magnetic Resonance Spectrum:

NMF (400 MHz, CD3OD, $\delta$):

3.36-3.40 (2H, m), 2.60.2.64 (2H, m), 1.78-1.87 (4H, m)

(6) $^{13}C$ Nuclear Magnetic Resonance Spectrum:

NMF (100MHz, CD3OD, $\delta$):

168.3 (s), 42.6 (t), 26.8 (t), 21.7 (t), 19.0 (t)

Additionally, when a determination of the structure was attempted from the physiochemical characteristics given above, the substance was identified as 2-iminopiperidine hydrochloride.

Continuation of the front page

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